UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of	: Linda H. Malkas
By the Examiner	: Huff, Sheela Jitendra
Docket No.	: 80371/5
Serial No.	: 10/083,576
Filed	: February 27, 2002
Group Art Unit	: 1643
Title	: METHOD FOR PURIFYING CANCER-SPECIFIC PROLIFERATING CELL NUCLEAR ANTIGEN

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION UNDER 37 C.F.R. §1.132

Dear Sir:

I, Linda H. Malkas, declare as follows:

1. I am a co-inventor, along with Robert J. Hickey, Pamela E. Bechtel, Lauren Schnaper, Min Park, Derek J. Hoelz, and Dragana Tomic, of the subject matter disclosed and claimed in the United States Patent Application Serial No. 10/083,576, filed February 27, 2002, entitled METHOD FOR PURIFYING CANCER SPECIFIC PROLIFERATING CELL NUCLEAR ANTIGEN.

2. I am a co-author of an article entitled *Detection of the Cancer Specific Form of PCNA by Elisa Assay*, Proceedings of the American Association For Cancer Research, Abstract No. 2507, vol. 42, p. 466 (EXHIBIT A) and am a co-inventor of the subject matter which is disclosed in this publication and disclosed and claimed in the above-referenced patent application.

3. In addition to myself, Robert J. Hickey, Lauren Schnaper, Derek J. Hoelz, and Dragana Tomic are co-inventors of the above-referenced patent application and co-authors of the above-referenced article.

4. P. Wills and C. Lankford are named as co-authors in the above-referenced article but are not inventors of the subject matter described in the above-referenced article or in the above-referenced patent application, but were listed as co-authors of the above-referenced article in order to receive credit for having collaborated in the research program by contributing to the program by providing materials.

5. P. Wills (Phil Wills) provided purified XPG-GST fusion protein for the ELISA.

6. C. Lankford (Carla Lankford) provided MCF7 (cancer) and MCF10A (Normal) cell extracts for the ELISA.

7. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-referenced application or any patent issuing thereon.

DATE: 8/9/06

Linda H. Malkas

EXHIBIT A

1. *Detection of the Cancer Specific Form of PCNA by Elisa Assay*, Proceedings of the American Association For Cancer Research, Abstract No. 2507, vol. 42, p. 466.

O^6-alkylguanine-DNA alkyltransferase (AGT). In vitro studies demonstrated that NNKOAc either directly, or as a result of DNA adduct formation, interferes with the repair O^6-mG by AGT. In this study, we show that NNKOAc depletes AGT in A/J mouse lung. We then compared the ability of NNKOAc and O^6-benzylguanine (O^6-bzG) to enhance the tumorigenic activity of AMMN in A/J mouse lungs. O^6-bzG is an established in vivo inhibitor of AGT. NNKOAc and O^6-bzG had similar effects on the levels of AMMN-derived O^6-mG at 4 and 96 h post-injection. NNKOAc and O^6-bzG enhanced the lung tumorigenic activity of a 0.75 μmol dose of AMMN to a similar extent. These data are consistent with the hypothesis that the pyridyloxobutylation pathway contributes to the lung tumorigenic activity of NNK in A/J mice by interfering with O^6-mG repair. To determine if AGT substrate pyridyloxobutyl adducts are present in lung DNA from NNK-treated mice, we measured the levels of O^6-[4-oxo-4-(3-pyridyl)-butyl]guanine(O^6-pobG) in lung and liver DNA 24 h after exposure to 10 μmol [5-^{3}H]NNK. This adduct was detected in liver but not lung DNA. The limits of detection were approximately 0.5 pmol O^6-pobG/μmol guanine. The implications of these findings will be discussed [Supported by CA-59887].

#2507 Detection of the Cancer Specific Form of PCNA by Elisa Assay. D. Tomic, D. J. Hoelz, P. Wills, R. J. Hickey, L. Schnaper, C. Lankford, and L. H. Malkas. *Greater Baltimore Medical Center, Towson, MD, and University of Maryland, Baltimore, MD.*

Proliferating cell nuclear antigen (PCNA) is a 36kD nuclear protein which is required for DNA replication (processivity factor of DNA polymerase δ) and DNA repair. Previously, using 2D-IEF-PAGE analyses, our laboratory discovered that malignant breast cells express a unique, acidic form of PCNA protein which can clearly be distinguished from the basic form of this protein found in non-malignant cells. Our research suggests that the acidic form of PCNA is, most likely, the result of a post translational modification. This finding is important because this unique form of PCNA in breast cancer cells could potentially serve as a powerful marker for the detection of this malignancy. Therefore, the purpose of this study was to develop an ELISA test, which can distinguish the malignant form of PCNA from the non-malignant form. We tested the hypothesis that xeroderma pigmentosum (XP)G protein, a structure-specific repair endonuclease similar to FEN1, and used in the nucleotide excision repair pathway is capable of distinguishing two forms of PCNA through binding affinities. To test this hypothesis, the protein isolated from the non-malignant breast cell line (MCF10A) and the breast cancer cell line (MCF-7) were used to measure the binding affinity of XPG to the acidic and basic form of PCNA in a modified ELISA assay. Standard curves, representing the correlation between absorbance and the abundance of the malignant and non-malignant form of PCNA were prepared and compared to each other. Serial dilutions of PCNA were tested in duplicate and the mean value of absorbance was calculated and used for comparison. Our results indicate that XPG protein has a different binding affinity for the malignant and non-malignant forms of PCNA. These results are the first to demonstrate that these two forms of PCNA can be distinguished by an ELISA assay that can be used clinically for the early detection of breast cancer.

#2508 Effects of Zinc Occupancy on the Function of Human O^6-Alkylguanine-DNA Alkyltransferase (AGT). Joseph J. Rasimas, Sreenivas Kanugula, Michael G. Fried, and Anthony E. Pegg. *Penn State College of Medicine, Hershey, PA.*

AGT is a small monomeric DNA repair protein whose homologs are found in a wide variety of prokaryotic and eukaryotic organisms. It is responsible for the repair of potentially mutagenic and cytotoxic alkyl and haloalkyl adducts of DNA, specifically at the O^6-position of guanine and, to a lesser degree, at the O^4-position of thymine. Unlike many proteins which are responsible for maintenance of genomic integrity, AGT is not an enzyme, but instead, restores DNA by irreversible transfer of adduct substituents to an internal active site sulfur atom (Cys145). Two crystal structures of human AGT (hAGT) have recently been published, and while both lend similar insight into the biophysical nature of this repair mechanism, they differ on one specific aspect of the protein's structure. One model suggests the presence of a zinc atom bound within a coordination sphere of at least four amino acid residues (Cys5, Cys24, His29, and His85) near the N-terminus, while the other model shows these residues in similar orientation, but lacking the transition metal ion. We have, therefore, begun to examine the structural and functional consequences of the relative occupancy of the protein's putative zinc binding site. In bacterial expression systems, recombinant hAGT is produced in increasingly larger quantities when growth media are supplemented with $ZnCl_2$ up to a concentration of 0.1 mM. Furthermore, metal-enriched hAGT samples with a molar zinc:protein binding ratio of 1.83 :1 (assessed by ICP-MS) demonstrate a 60-fold increase in repair rate constant over metal-stripped hAGT, as well as a 5-fold increase over conventionally purified protein samples with a ratio of 0.66:1. In addition, mutants of Cys5 and Cys24 (two of the putative zinc-binding residues) show 89% and 56% decreases in zinc occupancy compared to wild-type protein and repair methylated DNA substrate with activities of 17-fold and 3.5-fold less than wild-type AGT, respectively. Mutations and metal content manipulations have little or no effect upon the CD spectrum of hAGT proteins, suggesting that the overall structural fold of the protein is not modulated by the relative occupancy of the zinc site. Using an electrophoretic mobility shift assay with 16-mer oligonucleotides, differentially zinc-treated hAGTs and metal-binding residue mutants (C5A and C24A) also show the same affinity for binding to DNA. Repair deficient active s
occupancy show similar binding
ylguanine. We conclude, therefore, that while zinc is neither essential for DN repair by hAGT nor required for maintaining a functional fold of the protein, t presence of the transition metal ion bound within the polypeptide structu confers a mechanistic enhancement to repair activity which does not result fro an increase in substrate binding affinity. Zinc may also provide some measure structural stability to hAGT.

#2509 Molecular Alterations in the Transcription-Coupled Nucleotide Excision Repair Gene, CS-B/ERCC6, in Human Malignant Gliomas. Franc Ali-Osman, Kurt Jaeckle, Thomas Connor, Gamil Antoun, and Lixin Zhan *U.T. M.D. Anderson Cancer Center, Houston, TX.*

The CS-B/ERCC6 gene encodes a complementation factor required for effi cient transcription-coupled nucleotide excision repair (TC-NER), a major DN repair pathway by which a variety of lesions are removed from the cellula genome. In this study, we examined 39 primary human malignant glioma spec mens and their matched normal tissues, as well as, 11 early passage glioma ce lines, for molecular alterations (deletions and mutations) in the CS-B gene. Th results were correlated with the histological grade of the tumors. The resul showed, overall, CS-B gene deletions to increase with increasing glioma grad and exon II to be most frequently deleted exon. Frequencies of exon II deletion were 12.5%, 30.8% and 66.7%, in astrocytomas, anaplastic astrocytomas an glioblastoma multiformes, respectively. Mutation analysis performed by SSC analysis of exon II and confirmed by nucleotide sequencing, showed a lowe frequency of mutations in exon II of the CS-B gene in the tumors, with only 12.5% of the glioma, all anaplastic astrocytoma or glioblastoma multiforme, to harbo any mutations. The mutations were varied and comprised of nucleotide trans tions of CAC(H)→TAC(Y) in codon 13 and CAA(C)→CGG(H) in codon 15, an transversions of TCT(S)→TAT(Y) and CAG(Q)→CAC(H) in codons 57 and 7 respectively. These data suggest that defective TC-NER, resulting from genetic abnormalities in the CS-B gene, particularly, in exons 2 and 5, may contribute t malignant progression in gliomas.

#2510 Y-Box Binding Protein-1 Binds Preferentially to Single-Stranded Nucleic Acid and Exhibits 3' - 5' Exonuclease Activity. Hiroto Izumi, Toshihir Imamura, Gunji Nagatani, Tomoko Ise, Tadashi Murakami, Hidetaka Uramoto, Takayuki Torigoe, Hiroshi Ishiguchi, Yoichiro Yoshida, Minoru Nomoto, and Kimi toshi Kohno. *Department of Molecular Biology, University of Occupational and Environmental Health, Fukuoka, Japan, and University of Occupational and Environmental Health, Fukuoka, Japan.*

We previously have shown that YB-1 (Y-box binding protein-1) binds prefer entially to cisplatin-modified Y-box sequences. Based on structural and biochem ical data, we predicted that this protein binds single-stranded nucleic acids. In th present study we confirmed the prediction and also discovered some unexpected functional features of Y-box binding protein-1. We found that the cold-shock domain of the protein is necessary but not sufficient for double-stranded DNA binding while the C-tail domain interacts with both single-stranded DNA and RNA independently of the cold-shock domain. In an in vitro translation systems, th C-tail domain of the protein inhibited translation but the cold-shock domain did not. Antibodies recognizing the protein showed a supershift when single-stranded oligonucleotides were used as a probe, but not when double-stranded oligonu cleotides were used. Both in vitro pull-down and in vivo coimmunoprecipitation assays revealed that Y-box binding protein-1 can form a homodimer. Deletion analysis mapped the C-tail domain of the protein as the region of homodimer ization. We also characterized an intrinsic 3' - 5' DNA exonuclease activity of the protein. The region between residues 51 and 205 of its 324-amino acid extent is required for full exonuclease activity. Our findings suggest that Y-box binding protein-1 functions in regulating DNA/RNA transactions, and that these actions involve different domains and are influenced by dimerization of the protein.

#2511 Examining the Role of a Human 3'-5' Exonuclease (ExoN) In the Fidelity of DNA Polymerase α. Kevin R. Brown, Carole L. Galligan, and Violetta Skalski. *University of Toronto, Toronto, ON, Canada.*

3'-5' exonucleases are proteins that ensure the accuracy of DNA replication by catalyzing the removal of mispaired nucleotides from the 3'-termini of nascent DNA. Base mutations arising through nucleotide misinsertions are considered to be important contributors to the development of cancer. Of the polymerases thought to be involved in DNA replication, only DNA polymerase alpha (pol α) lacks an intrinsic proofreading exonuclease, although it has been suggested that an unassociated 3'-5' exonuclease may provide this function. We have purified a previously uncharacterized 3'-5' exonuclease (exoN) from the nuclei of either primary or established acute myeloblastic leukemia (AML), chronic lymphocytic leukemia (CLL), and human cervical tumor cells, as well as normal peripheral blood lymphocytes. The apparent ubiquitous nature of exoN suggests this exonuclease has a significant biological function. ExoN is a 46kDa monomer, and is active on single- and double-stranded DNA. Kinetic studies have shown that exoN binds very tightly to duplex DNA substrates, with a Km of 0.3nM (correctly base-paired 3'-ends). In this study, we have set out to examine the impact of exoN on the fidelity of the exonuclease-deficient mammalian DNA polymerase α. Using an in vitro fidelity assay, we have examined the removal of all 16 possible nucleotide pairs. ExoN was shown to efficiently remove mispaired nucleotides

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2. I am a co-author of an article entitled *An Altered Form of Proliferating Cell Nuclear Antigen in Various Cancers* (EXHIBIT A), that was included as part of the Doctoral Dissertation of Pamela E. Bechtel entitled *Proliferating Cell Nuclear Antigen in Malignancy,* that was submitted to the Faculty of the Graduate School of the University of Maryland in 1998 and approved May 8, 1998, and am a co-inventor of the subject matter which is disclosed in this publication and disclosed and claimed in the above-referenced patent application.

3. In addition to myself, Robert J. Hickey and Pamela E. Bechtel are co-inventors of the above-referenced patent application and co-authors of the above-referenced article.

4. Lori N. Croisetiere, Brian J. Long, Moshe Talpaz, and Lawrence Chin are named as co-authors in the above-referenced article but are not inventors of the subject matter described in the above-referenced article or in the above-referenced patent application, but were listed as co-authors of the above-referenced article in order to receive credit for having collaborated in the research program by contributing to the program.

5. Lori N. Croisetiere was a technician in the laboratory helping Pamela E. Bechtel with her project.

6. Brian J. Long was a post doc who supplied primary breast cells from Dr. Angela Brodie's laboratory.

7. Moshe Talpaz was a division chief at MD Anderson and provided sera from Chronic Myelogenous Leukemia patients under his care and from several of his residents/fellows who served as normal controls for the CML patients.

8. Lawrence Chin provided ovarian cancer tissue.

9. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the above-referenced application or any patent issuing thereon.

DATE: 9/9/06

Linda H. Malkas

EXHIBIT A

1. *An Altered Form of Proliferating Cell Nuclear Antigen in Various Cancers*, (Taken from Doctoral Dissertation of Pamela E. Bechtel entitled *Proliferating Cell Nuclear Antigen in Malignancy,* that was submitted to the Faculty of the Graduate School of the University of Maryland in 1998 and approved May 8, 1998).

Title: An Altered Form of Proliferating Cell Nuclear Antigen in Various Cancers.

Authors: Pamela E. Bechtel, Robert J. Hickey, Lori N. Croisetiere, Brian J. Long, Moshe Talpaz, Lawrence Chin and Linda H. Malkas

Key Words: PCNA, tumor marker, post-translational modification, epigenetic change, breast cancer, prostate cancer, malignant glioma, leukemia, cervical cancer, colon cancer

ABSTRACT

The process transforming benign cells into malignant cells involves a complex series of molecular and structural alterations which are poorly understood. One characteristic common to most malignancies is the aberrant proliferation of cells. Cellular proliferation is regulated at a number of points throughout the cell cycle through complex interaction of many proteins. Together these proteins form checkpoints that restrict the ability of cells to progress through the cell cycle. Several critically important checkpoints monitor the cells prior to the entrance into S phase. These checkpoints ultimately signal the DNA synthetic apparatus to begin DNA replication. A multiprotein DNA replication complex, DNA synthesome, has been isolated and characterized from human cells and found to be fully competent to mediate DNA replication *in vitro*. Proliferating cell nuclear antigen (PCNA) was found to be a component of the DNA synthesome. PCNA is an accessory protein for polymerase δ and is involved in DNA repair. A recent study has identified a unique form of PCNA in malignant breast cells that significantly differs in isoelectric point (pI) from the PCNA found in nonmalignant breast cells. The purpose of this study was to determine whether different types of malignant cells contain the altered form of PCNA. To examine this possibility, the DNA synthesome was isolated from malignant prostate cells (LNCaP, PC50), malignant colon cells (KGE90, KYE350, SW48), cervical cancer cells (HeLa), malignant brain cells (T98) and leukemia cells (HL60, CML, AML). The components of the synthesome were resolved by 2 dimensional gel electrophoresis (2D PAGE). Western blot analysis was performed using an antibody directed against PCNA. The results of the analysis demonstrated that the altered form of PCNA was present in all of the malignant cells examined. These findings suggest that the structural alteration of PCNA

may be involved in the development of malignancies due to the critical role PCNA has in the DNA replication and repair processes.

INTRODUCTION

Cancer is the second leading cause of death in the United States (Parker *et al.*, 1997). Lung cancer alone was responsible for more than 160,000 cancer related deaths in 1997, and it is the leading cause of cancer mortality (Parker *et al.*, 1997). Among men, prostate cancer is the second leading cause of cancer related deaths and more than 300,000 new cases were diagnosed in 1997 (Parker *et al.*, 1997). Breast cancer is the second leading cause of cancer related deaths among women and approximately 180,000 new cases of breast cancer were diagnosed in 1997 (Parker *et al.*, 1997). Colon cancer is also a leading cause of cancer deaths in men and women, with 46,000 deaths attributed to this malignancy in 1997 (Parker *et al.*, 1997; Weinberg and Strom, 1995). Leukemias and brain cancers are the most common childhood malignancies with acute lymphocytic leukemia accounting for 80% of childhood cancers (Parker *et al.*, 1997; Kumar *et al.*, 1992).

The origin and clinical manifestations of a malignancy differ for each individual cancer. Breast cancers are usually ductal or lobular in origin and generally affect post menopausal women (Dickson and Lippman, 1992). Prostate and colon cancers generally arise from benign lesions which progress into malignancies. Usually these forms of cancer affect people over the age of fifty (Isaacs, 1997; Meyers and Grizzle, 1997; Parker *et al.*, 1997; Johnson, 1995; Weinberg and Strom, 1995). Many brain tumors arise from astrocytes and are generally refractory to chemotherapy (Kordek *et al.*, 1996; Dalrymple *et al.*, 1994). Cervical cancer has been shown to be caused by human papillomavirus (HPV), and generally occurs in women over the age of thirty five (Villa, 1997; Parker *et al.*, 1997). Leukemias, in contrast to solid tumors, arise in the bone marrow and result in the circulation of immature white cells in the peripheral blood (Kumar *et al.*, 1992). Leukemia can affect people of all ages but children

with leukemias have a significantly increased survival rate compared to adults (Parker *et al.*, 1997; Kumar *et al.*, 1992).

The diagnosis of cancer at an early developmental stage is important in reducing cancer mortality. The use of mammography to detect developing breast cancers has reduced the mortality rate of the disease (Hayes, 1996). Testing for the presence of prostate specific antigen (PSA) has enabled clinicians to diagnose prostate cancer in men early in its onset (Small, 1997; Gao *et al.*, 1997). As a marker, PSA has high sensitivity and specificity and correlates with tumor burden (Pandha and Waxman, 1995; Magdelenat, 1992). However, a major limitation is the inability to distinguish between benign and malignant prostate lesions by evaluating PSA (Pandha and Waxman, 1995; Magdelenat, 1992). Routine Pap smear screens have been routinely used for the past twenty years and can be beneficial for the early detection and monitoring of cervical cancer among women. Although screening methods exist to detect certain types of malignancy the basic mechanisms for the development of cancer have not been determined.

One commonality of virtually all malignancies is the aberrant proliferation of cells. Aberrant cell proliferation is presumably due to escape from the normal controls which regulate the progression of the cell into specific stages of the cell cycle. Recently, an altered form of the protein proliferating cell nuclear antigen (PCNA) was identified from malignant human breast cells (Bechtel *et al.*, submitted). PCNA is a nuclear protein that is required for DNA synthesis and DNA repair. In this report, PCNA was analyzed from a variety of human malignancies to determine whether the unique form of PCNA was present only in malignant breast cells or whether it was present in other types of malignant cells. Several malignant human cell lines were used: LNCaP and PC50 (prostate adenocarcinoma); KGE90, KYE350 and SW48 (colon adenocarcinoma); T98

(malignant glioma); HeLa (cervical carcinoma); and HL60 (promyelogenous leukemia). PCNA was also analyzed from chronic myelogenous leukemia specimens, acute myelogenous leukemia sample and serum collected from cancer free individuals.

METHODS

Cell culture: HeLa cells were maintained in Dulbucco's Modified Eagles Medium (DMEM) with 10% bovine calf serum. HL60 and LNCaP cells were maintained in RPMI 1640 media with 10% fetal bovine serum. PC50 cells were maintained in Ham's F12K media with 7% fetal bovine serum. KGE90, KYE350 and SW48 cells were maintained in L-15 medium with 10% bovine calf serum. T98 cells were maintained in Eagles MEM with nonessential amino acids, 1.0 mM Sodium pyruvate and Earles BSS, and 10% fetal bovine serum.

DNA synthesome isolation: The DNA synthesome was isolated from LNCaP, PC50, KGE90, KYE350, HL60, HeLa, T98 and leukemia cell pellets according to published procedures (Coll *et al.*, 1996) with minor modifications. Briefly, cell pellets were Dounce homogenized in a buffer containing 200 mM sucrose, 50 mM Hepes, 5 mM KCl, 2 mM DTT and 0.1 mM PMSF and centrifuged at 2500 rpm for 10 min to remove cell debris and pellet the nuclei. EDTA and EGTA were added to a final concentration of 5 mM to the supernatant. One volume of nuclear extraction buffer (350 mM KCl, 50 mM Hepes, 5 mM $MgCl_2$, 5 mM EDTA, 5 mM EGTA, 1 mM DTT, 0.1 mM PMSF) was added to the pellet and rocked at 4°C for 2 hr. The supernatant was centrifuged at 18,000 rpm for 3 min. The supernatant was removed and centrifuged at 60,000 rpm for 22 min. The resulting supernatant was collected (S3 fraction). The nuclear pellet was centrifuged at 22,000 rpm for 3 min. The supernatant was removed and

centrifuged at 60,000 rpm for 15 min. The resulting supernatant (NE fraction) was combined with the S3 fraction, layered on top of a 2 M sucrose cushion and centrifuged overnight at 40,000 rpm. The synthesome fraction was collected for analysis.

2 Dimensional polyacrylamide gel electrophoresis (2D PAGE): DNA synthesome protein (20-40 μg) was loaded onto the first dimension tube gel (9.2 M urea, 4% acrylamide, 2% ampholytes (pH 3-10), and 20% Triton X-100). The polypeptides were separated along a pH gradient established using 100 mM NaOH and 10 mM H_3PO_4. The tube gels were placed onto an 8% acrylamide SDS gel, and the polypeptides were resolved by molecular weight. The proteins were then transferred electrophoretically to nitrocellulose membranes.

Western blot analysis: An antibody directed against PCNA (PC 10, Oncogene Science) was used at a dilution of 1:1000. Immunodetection of PCNA was performed using a light enhanced chemiluminescence system (Amersham).

Leukemia samples: Chronic myelogenous leukemia (CML) samples were obtained from Dr. Moshe Talpaz through a collaboration with the MD Anderson Cancer Center. The acute myelogenous leukemia (AML) sample was obtained from Dr. Lynn Abruzzo through a collaboration with the Greenebaum Cancer Center.

RESULTS

PCNA in Malignant Prostate Cells

The DNA synthesome was isolated from LNCaP and PC50 prostate adenocarcinoma cell lines and the components of the synthesome were resolved by two dimensional gel electrophoresis (2D PAGE). Western blot analysis was performed using an antibody directed against PCNA to determine whether the acidic form of PCNA was present in the malignant prostate cells. The acidic form of PCNA was readily detected in both of the prostate cancer cell lines examined (Figure 1).

PCNA in Malignant Colon Cells

PCNA associated with the DNA synthesome was isolated from three colon adenocarcinoma cell lines, KGE90, KYE350 and SW48. The components of the synthesome were resolved by 2D PAGE followed by Western blot analysis using an antibody directed against PCNA. The results of this analysis indicate that the three colon cancer cell lines contained the acidic form of PCNA (Figure 2). Two of the cell lines (KGE90 and KYE350) only contained the acidic form of PCNA while the SW48 cell line contained both forms of PCNA.

PCNA in Malignant Brain and Cervical Cells

PCNA was isolated from a malignant glioma cell line (T98) and a malignant cervical cancer cell line (HeLa) to determine whether the altered form of PCNA was present. 2D PAGE and Western blot analyses were performed using a PCNA specific antibody. Figure 3A shows that the altered form of PCNA was present in HeLa cells. Figure 3B illustrates that T98 cells contained the acidic form of PCNA.

PCNA in Leukemia

Blood samples from patients with chronic myelogenous leukemia (CML) and acute myelogenous leukemia (AML), serum collected from cancer free individuals, and a human leukemia cell line (HL60) were analyzed to determine whether the acidic form of PCNA was present. The DNA synthesome was isolated from the leukemia samples and the HL60 cell line and the components resolved by 2D PAGE followed by Western blot analysis using an antibody directed against PCNA. The protein migration pattern exhibited by PCNA is shown in Figure 4. The acidic form of PCNA was identified in HL60 cells (Figure 4A), the AML sample (Figure 4B) and all of the CML samples (Figures 4C-E). The acidic form of PCNA was not detectable in the serum collected from cancer free individuals.

DISCUSSION

The diagnosis of cancer during its earliest stages of development is important in reducing cancer related mortality. Currently, a major limitation in the diagnosis of most cancers is the inability to reliably detect tumors prior to the onset of clinical manifestations. The identification of specific cellular transformation events that occur during the initial phase of tumor development may be beneficial in identifying novel tumor markers to better detect and monitor malignancies. A specific molecular alteration was recently identified in breast cancer cells which has the potential to serve as a breast cancer tumor marker (Bechtel *et al.*, submitted). To determine whether the altered form of PCNA was also present in other types of cancer cells, several different human malignancies were evaluated.

Analysis of prostate cancer cells in this study demonstrated the presence of the altered form of PCNA. Previous reports indicated that PCNA is strongly expressed in prostate cancers and also has been detected in dysplastic luminal cells of intraepithelial neoplasia, a premalignant prostate lesion (Pandha and Waxman, 1995; Magdelenat, 1992). Gao *et al.* (1997) reported that PCNA levels correlate with the clinical stage and metastatic state of prostate tumors. The researchers suggested that PCNA levels have the potential to be used as a prognostic marker for prostate cancer (Gao *et al.*, 1997).

The colon cancer cell lines examined in this study also contained the altered form of PCNA. Studies have shown that PCNA labeling index is correlated with tritiated thymidine uptake in assessing the proliferative activity of colon tumors (Bleiberg *et al.*, 1993). Neoptolemos *et al.* (1995) demonstrated that PCNA levels used in conjunction with the Duke's classification system (the classification system for colon tumors based on histological examination) improved the prediction of survival for colon cancer patients. Mitchell *et al.* (1997) demonstrated that increased levels of PCNA are found in primary colon tumors and in resulting lung metastases. It has been shown that PCNA levels increase during the progression of benign lesions to malignancy and that increased levels are associated with the recurrence of adenomas (Shpitz *et al.*, 1997; Paspatis *et al.*, 1994; Risio *et al.*, 1993). Risio *et al.* (1993) hypothesized that the increase in the number of PCNA labeled cells is related to hyperproliferation as well as to the neoplastic deregulation of PCNA synthesis in the development of colon cancer.

Malignant gliomas are composed of heterogeneous subpopulations of cells with different cytogenetic compositions making it difficult to identify useful prognostic markers (Dalrymple *et al.*, 1995). Previous studies demonstrated that PCNA labeling index correlates with the histological grade of the brain

malignancy. In the majority of benign astrocytomas, the PCNA labeling index is low (Kordek *et al.*, 1996). Kordek *et al.* (1996) also have hypothesized that malignant gliomas contain two forms of PCNA, one of which does not have a role in DNA synthesis. The results of the present study have indicated that malignant glioma cells contain the altered form of PCNA. The functional role of the altered form of PCNA needs to be examined in greater detail to determine whether it has a role in the development and progression of malignant gliomas.

The role of PCNA in the development and progression of CML is not well characterized. CML is a biphasic disease characterized by an early chronic phase followed by a blast phase (Zaccaria *et al.*, 1995). Takasaki *et al.* (1984b) demonstrated a correlation between the number of leukocytes expressing PCNA and the percent of blast cells in blood during the blast phase of CML. These investigators also identified the presence of non-blast cells which were positive for PCNA in the peripheral blood during the blast phase of CML. This result differs from the observation that the non-blast cells were negative for PCNA in the chronic phase (Takasaki *et al.*, 1984). The PCNA labeling index for CML cells is not significantly different form normal bone marrow cells (Thiele *et al.*, 1993). However, in the chronic myeloid proliferative disorder osteomyelofibrosis, there is a significant increase in the PCNA labeling index (Thiele *et al.*, 1994). Interferon treatment resulted in decreased PCNA labeling. In the present study, the results demonstrated that the leukemia samples examined contain the altered form of PCNA, while samples collected from cancer free individuals did not contain the altered form of PCNA.

Analysis of HeLa cells in the present study revealed that these cervical cancer cells contain the altered form of PCNA. Cardillo *et al.* (1993) found that PCNA levels were increased in all cases of invasive squamous carcinoma, cervical adenoma, cervical carcinoma in situ (CIS) and cervical intraepithelial

neoplasia (CIN) III examined. Smela *et al.* (1984) demonstrated a significant correlation between PCNA index and the severity of cervical dysplasia by determining the PCNA levels in CIN I, CIN II and CIN III lesions. Other studies found that PCNA index was increased in premalignant and malignant cervical lesions compared to nonmalignant tissue (Steinbeck *et al.*, 1995; Raju *et al.*, 1994; Shurbaji, 1993). Kobayski *et al.* (1994) concluded that the increase in PCNA labeling index suggests that there is considerable alteration of biologic activity, including genetic mutations, during the progression of severe cervical dysplasia to CIS.

This study examined several human malignancies for the presence of the acidic form of PCNA. The malignancies examined in this study have few common characteristics or clinical manifestations. Previously, an altered form of PCNA was identified in malignant breast cells. It was demonstrated that the unique form of PCNA was not the result of growth stimulation or genetic mutation (Bechtel *et al.*, submitted). Further research indicated that differential post-translational modification by poly(ADP-ribosylation) was at least partially responsible for the presence of the altered form of the protein (Bechtel *et al.*, submitted). This report identified the acidic form of PCNA in leukemia, prostate, cervical, colon and brain cancers. These data demonstrated a simple molecular alteration in the PCNA polypeptide is a common feature between these diverse types of cancer. These findings suggest that the acidic form of PCNA may have an underlying role in the development of different types of cancer and that these cancers may develop as a consequence of a global alteration in the manner in which specific cellular proteins are post-translational modified.

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FIGURE LEGENDS

Figure 1: The protein migration pattern of PCNA from malignant prostate cells. The DNA synthesome was isolated from LNCaP and PC50 prostate cancer cells. The components of the synthesome were resolved by 2D PAGE, and subjected to Western blot analyses using an antibody directed against PCNA. The electrophoretic mobility of PCNA is shown for (A) LNCaP cells and (B) PC50 cells. The arrow indicates the position of the acidic form of PCNA.

Figure 2: The protein migration pattern of PCNA from malignant colon cells. The DNA synthesome was isolated from KGE90, KYE350 and SW48 colon cancer cells. The components of the synthesome were resolved by 2D PAGE and subjected to Western blot analyses using an antibody directed against PCNA. The electrophoretic mobility for PCNA is shown for (A) KGE90 cells, (B) KYE350 cells and (C) SW48 cells. The arrow indicates the position of the acidic form of PCNA.

Figure 3: The protein migration pattern of PCNA from malignant brain and cervical cells. The DNA synthesome was isolated from malignant glioma (T98) and cervical (HeLa) cells. The components of the synthesome were resolved by 2D PAGE and subjected to Western blot analyses using an antibody directed against PCNA. The electrophoretic mobility for PCNA is shown for (A) HeLa cells and (B) T98 cells. The arrow indicates the position of the acidic form of PCNA.

Figure 4: The protein migration pattern of PCNA from leukemia cells. The DNA synthesome was isolated from HL60 cells, CML samples, an AML sample

and serum collected from cancer free individuals. The components of the synthesome were resolved by 2D PAGE and subjected to Western blot analyses using an antibody directed against PCNA. The electrophoretic mobility for PCNA is shown for (A) HL60 cells, (B) AML cells and (C-E) CML cells. The arrow indicates the position of the acidic form of PCNA.

Figure 1

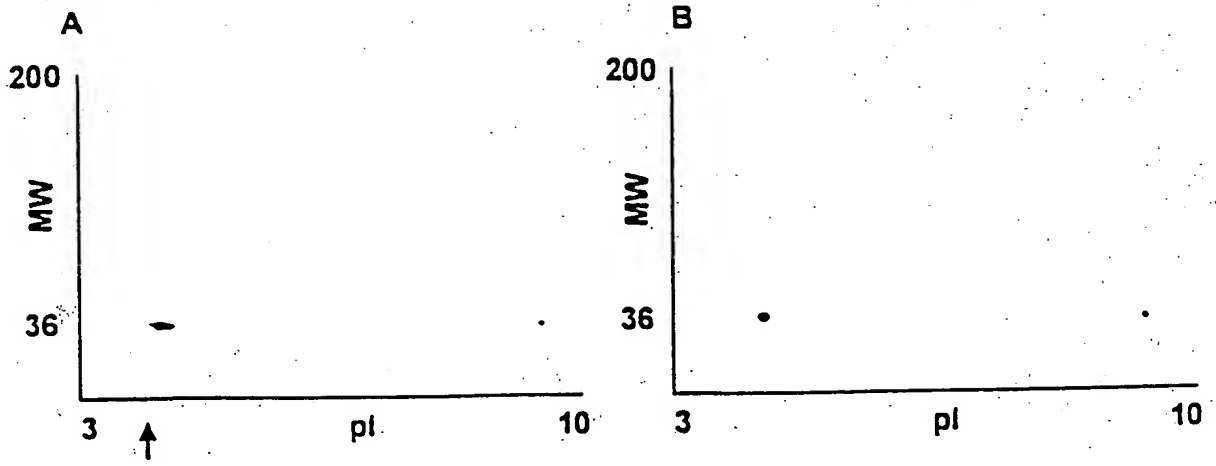


Figure 2

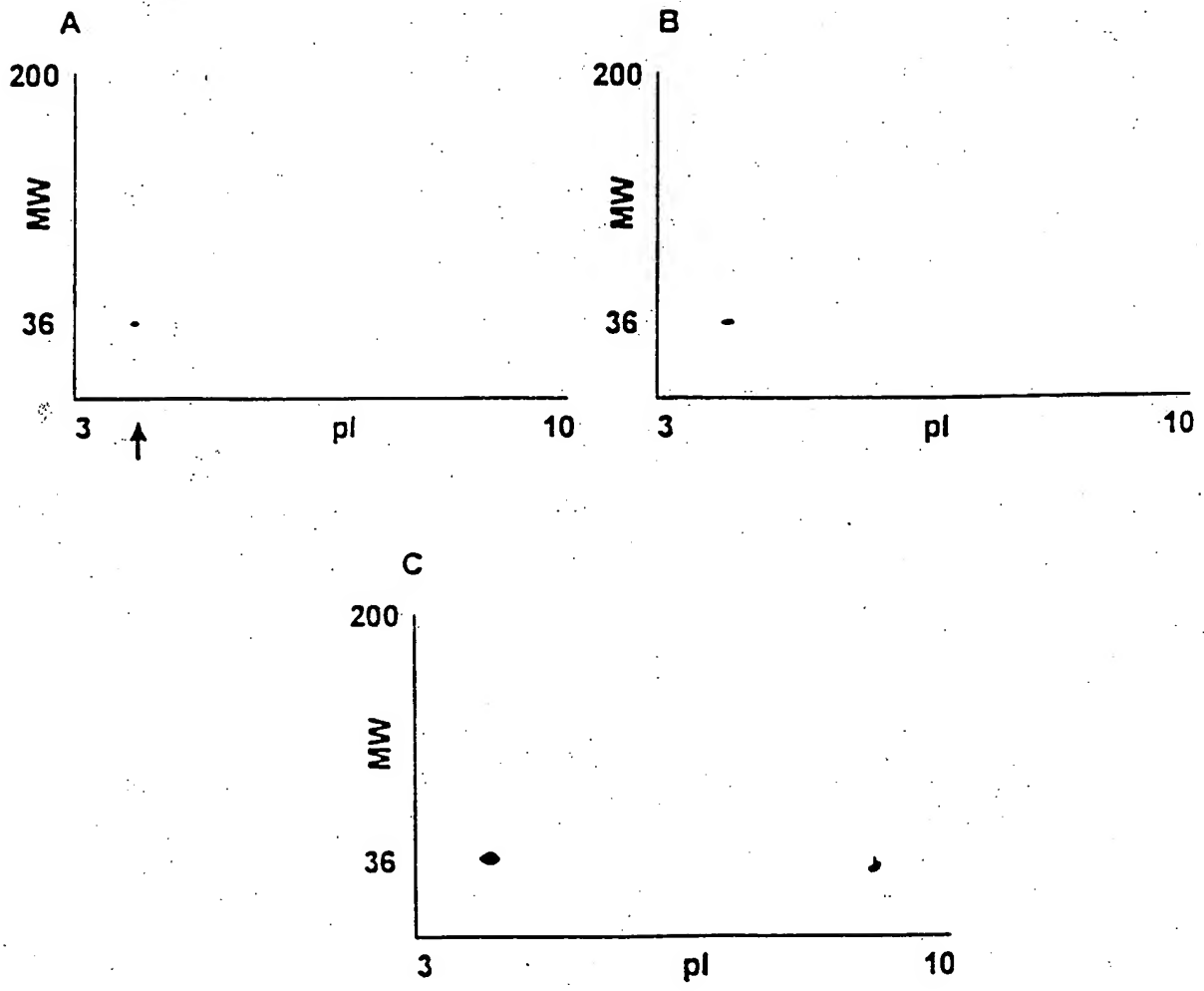


Figure 3

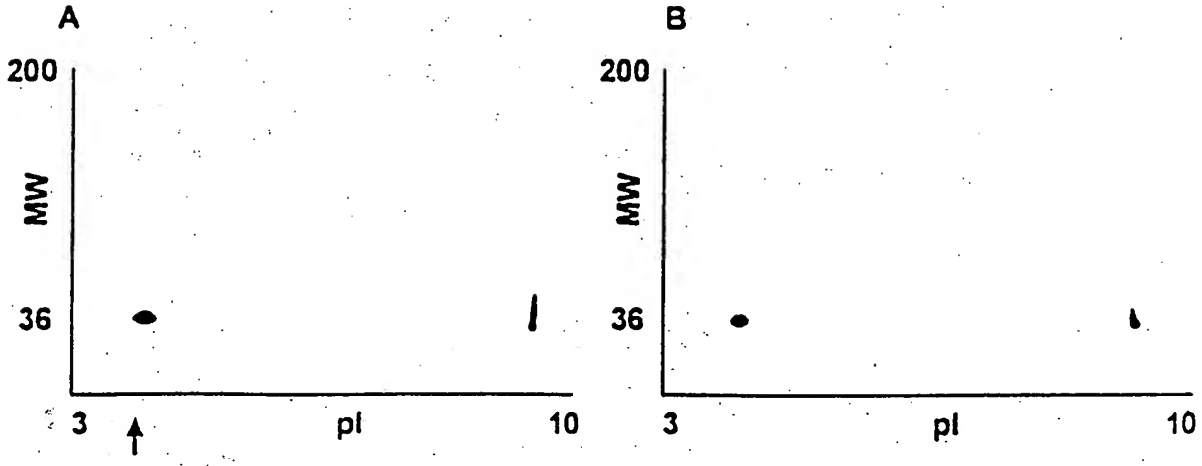
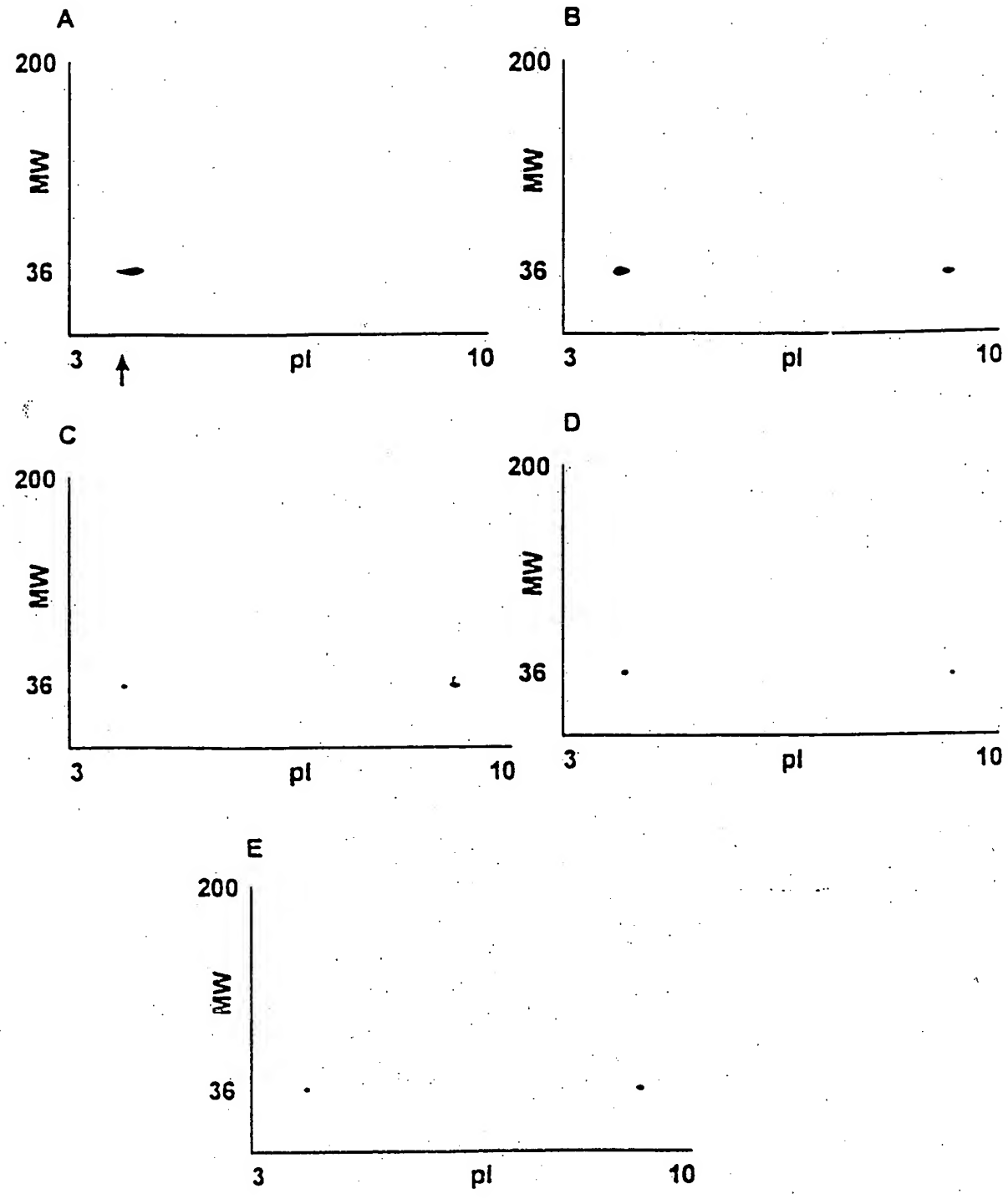


Figure 4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